Reference: *Biol. Bull.* **208:** 200–212. (June 2005)


# Gill Anatomy and the Evolution of Symbiosis in the Bivalve Family Thyasiridae

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**Abstract.** Among families of bivalves with chemoautotrophic symbionts, the Thyasiridae may vary the most in their anatomical characters and in the extent of their nutritional reliance upon symbionts. Since only a fraction of thyasirid species are symbiotic, and the symbionts are mostly observed to be extracellular, this group may be representative of early stages in the evolution of bacterium-bivalve symbioses. To better understand the distribution of symbiosis among thyasirid genera, and the relationships between gill structure and symbiont occurrence, the gills of 26 thyasirid species were studied by light and electron microscopy. Observations revealed three gill types, which are generally constrained within genera or subgenera. Symbionts were found in two gill types: the most simple, homorhabdic filibranch morphotype, and the most derived and thickened morphotype, which resembles the gill structure of other chemosymbiotic bivalves. In all observable cases, the symbionts were located extracellularly among the microvilli of the bacteriocytes. Among individuals of the species *Thyasira* (*Parathyasira*) *equalis*, the quantity of symbionts varied. The results suggest an evolutionary sequence: a homorhabdic filibranch gill structure with few symbionts among the epithelial cell microvilli eventually thickened abfrontally, and thereby offered a larger surface for colonization by symbionts. Eventually, the symbionts persisted and grew in vacuoles within epithelial cells.

## INTRODUCTION

Among the families of bivalves that receive a nutritional benefit from their association with symbiotic, chemoautotrophic bacteria (see Le Pennec *et al.*, 1995, for review), the Thyasiridae (subclass Heterodonta) stand out in several ways. (1) Whereas the bacteria in all other known bivalves are endosymbionts, those of studied thyasirids (with the exception of *Maorithyas hadalis*, see Fujiwara *et al.*, 2001) are extracellular (Southward, 1986). (2) Thyasirid symbionts that have been phylogenetically characterized fall into separate clades, rather than clustering as in most other groups of chemosymbiotic bivalves (Imhoff *et al.*, 2003). (3) Only within the Thyasiridae is there a genus, *Thyasira*, in which some species contain symbionts and others lack them (Southward, 1986). (4) Thyasirids have a much wider distribution than other chemosymbiotic bivalve families; they are found from coastal to hadal depths, in different types of sediments, and from both poles to the equator. However, the distribution of symbiotic thyasirids may be more restricted. (5) Thyasirids, as opposed to many other bivalves with chemoautotrophic symbionts, are small; most are less than a centimeter long.

The gills of thyasirids are also distinctive. The family Thyasiridae is the only one in which demibranch number is a variable character. Whereas the outer demibranch is reduced in some thyasirids, it is absent in others; this absence may be explained by paedomorphy (Stasek, 1963; Reid and Brand, 1986; Payne and Allen, 1991). Thyasirid gills also vary in the extent to which the filaments are expanded abfrontally (Southward, 1986; Payne and Allen, 1991). Typically, bivalves with symbiotic chemoautotrophic bacteria have modified gill filaments, in which the abfrontal end is expanded, its epidermis is increased in area and thickness, and the cells, called bacteriocytes, are modified to house the symbionts (Distel, 1998). Some thyasirids with symbionts have such expanded gill filaments, with a bacteriocyte zone similar to that of some lucinids (Southward, 1986). But the filaments in one symbiotic species, *Thyasira equalis*, are

Received 21 June 2004; accepted 29 March 2005.

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thin, transparent, and unmodified (Southward, 1986); thus they resemble, in transverse section, many homorhabdic filibranch gill filaments. Because symbiotic thyasirids have gills with variable degrees of abfrontal differentiation, the family provides a unique opportunity for addressing evolutionary questions related to symbiosis.

The relationship between the structure of thyasirid gills and the occurrence of bacterial symbionts has, in fact, been described in seven species (Southward, 1986; Le Pennec *et al.*, 1988; Fujiwara *et al.*, 2001). In this study, to explore these relationships more thoroughly, gill structure and symbiont occurrence were examined in 26 thyasirid species, either freshly collected or obtained from museum collections, and varying, not only in anatomical characters, but also in size and habitat. Given the paucity of phylogenetic data on this family and the anatomical variability within this group, we must consider that the Thyasiridae may be polyphyletic. Even so, the patterns of variation in gill complexity and symbiont location found within thyasirids may suggest the gradual changes that can lead to endosymbiosis in epithelial cells.

## MATERIALS AND METHODS

Live specimens and specimens obtained from museum collections were used in this study (Table 1). The nomenclature used follows Oliver and Killeen (2002); therefore, *Axinulus* and *Mendicula*, which other authors have determined to be subgenera of *Thyasira*, are here considered to be genera. The taxonomic affiliation of some species being unclear, they were classified with the taxon which they most resemble in shell character and demibranch number.

Within an hour after collection, live specimens were removed from their shells, and the gills were fixed for a minimum of 1 h in 3% glutaraldehyde in 0.1 *M* sodium phosphate buffer (pH 7.3) with 0.35 *M* sucrose. The tissues were then rinsed in the 0.1 *M* sodium phosphate buffer and postfixed for 1 h in 1% osmium tetroxide in the same buffer. After fixation, the gills were dehydrated in an ethanol gradient and embedded in Spurr resin.

Most of the specimens obtained from museum collections had been immersed in formaldehyde with their shells closed, so the gill epithelia were in a relatively poor state of preservation. However, these specimens could still provide useful information about gill anatomy, and symbionts could sometimes be detected and located. The gills of these specimens were removed and processed in the same manner as the live specimens.

Semi-thin (1–2 $\mu$m), transverse sections of the gills of all specimens were stained with toluidine blue, and ultra-thin sections (approximately 70 nm) were stained with uranyl acetate for 15 min, and with lead citrate for 7 min. Observations and micrographs were made on a LEO EM 912 or a Philips 410A transmission electron microscope.

A few museum specimens from the San Diego Trough (*Conchocele excavata, Thyasira (Thyasira)* sp., and *Adontorhina cyclia;* Table 1) were prepared for scanning electron microscopy. Following the removal of one valve, the specimens were post-fixed in 2.5% glutaraldehyde in a 0.1 *M* sodium cacodylate buffer (osmolarity $\approx$ 1050 mosmol). The half-shell preparations were dehydrated in an ascending ethanol gradient, critical-point-dried from $CO_2$, mounted on stubs, and sputter-coated with a gold/palladium mixture. Observations were made on a Cambridge Instruments 350 scanning electron microscope.

When bacteria were observed in association with abfrontal surfaces of a given species, they were assumed to be symbionts if they were abundant, found on most abfrontal cells, and on more than one individual of a species (ideally, from different collection sites). In four species, symbiont presence was inferred from observations of only one individual; but those were species with a highly derived gill structure which, in all other observed cases, represented an adaptation to symbiosis.

## RESULTS

The semi-thin gill sections revealed wide variations in gill filament morphology within the family (Fig. 1). Thyasirid gills have either a homorhabdic filibranch design (type 2) or a structure derived from it, with more interfilamentar tissue fusion in the abfrontal area (type 1), or with filaments expanded abfrontally and extensive epithelial surfaces accomodating bacteria (type 3).

### *Gill type 1*

Only the three species in the genus *Axinopsida* had gill type 1 (Table 2). This type is characterized by filaments without abfrontal expansion and with a high degree of inter-filamentar fusion throughout most ($\sim$ 70%) of the dorsoventral length of the filaments (Fig. 1a). This extensive fusion precludes water flow through the gill, except in the dorsalmost part where filaments are separate (as in Fig. 1b), allowing the flow of water from the infrabranchial to the suprabranchial chambers. In the highly fused areas of the gill, water currents must be parallel to the dorsoventral axis of the filaments; they are likely to be directed dorsalward, as in all suspension-feeding bivalves examined (Beninger and St-Jean, 1997). The tissue joining individual filaments contains many mucocytes (Fig. 1a, 2), which may secrete to the frontal surface *via* ducts, as in some lucinids (Duplessis *et al.*, 2004). In one of four specimens of *Axinopsida serricata, Rickettsia*-like nodules were seen, both in the abfrontal area and among the lateral and laterofrontal ciliated cells (Fig. 2). No bacterial symbionts were observed in the specimens with gill type 1.

**Table 1**

*Summary of collection information for thyasirid specimens examined*

| Species[1] | $n$[2] | Size (mm) | Collection site[3] | Depth (m) |
|---|---|---|---|---|
| Specimens collected in the field | | | | |
| *Axinulus croulinensis* | 1 | 2.2 | Raunefjord, Norway (60° 16.235′N, 5°08.631′E) | 220–253 |
| *Mendicula ferruginea* | 2 | 2–3 | Raunefjord, Norway | 220–253 |
| *Thyasira (?) obsoleta* | 5 | 2–3 | Raunefjord, Norway | 220–253 |
| *Thyasira (Parathyasira) equalis* | 71 | 2–5 | Raunefjord, Norway | 220–253 |
| *Thyasira (Thyasira) sarsi* | 49 | 3–8.5 | Dolviken, Norway (60°19.185′N, 5°15.344′E) | 50–54 |
| *Thyasira (Thyasira) flexuosa* | 61 | 3–9 | Dolviken, Norway | 50–54 |
| | 46 | 2–6 | La Coruña, Spain (43°21.449′N, 8°22.404′W, 43°21.880′N, 8°23.220′W) | 13–16 |
| | 2 | 4–6 | Long Beach, CA, USA (33°40.886′N, 118°19.313′W) | 151 |
| Specimens from museums | | | | |
| *Axinopsida orbiculata* | 2 | 2–5 | Tatar Strait[A] | 30 |
| | 3 | 2 | E. Greenland[B] | 3–9 |
| *Axinopsida serricata* | 2 | 4 | Bering Sea[C] | 36 |
| | 2 | 5 | Vancouver Island[D] | 58–190 |
| *Axinopsida subquadrata* | 1 | 4.5 | Okhotsk Sea[A] | 25 |
| | 1 | 4.5 | Japan Sea[A] | 48 |
| *Axinulus croulinensis** | 2 | 2 | North Sea[B] | 200 |
| *Axinulus eumyaria* | 3 | 2 | North Sea oil field[B] | 300–350 |
| *Axinulus* sp. | 1 | 1 | Doubtful Sound, NZ[E] | 376 |
| *Mendicula ferruginea** | 1 | 3 | North Sea[B] | 200 |
| *Mendicula pygmaea* | 1 | 2 | North Sea[B] | 200 |
| *Adontorhina cyclia* | 2 | 2 | San Diego Trough[F] | 1199–1250 |
| *Genaxinus debilis* | 1 | 1 | South Orkney Islands[C] | 298–403 |
| | 1 | 2 | Anvers Island, Antarctica[E] | 18 |
| *Genaxinus* sp. | 1 | 2 | South Orkney Islands[C] | 298–403 |
| *Thyasira (?) obsoleta** | 2 | 2–3 | North Sea[B] | 200 |
| | 1 | 2 | Off Beaufort, NC[G] | 198 |
| *Thyasira (Parathyasira) granulosa* | 1 | 7 | North Sea oil field[B] | 300–350 |
| *Thyasira (Parathyasira) equalis** | 2 | 3 | Barents Sea[A] | 195–380 |
| | 3 | 4 | North Sea[B] | 200 |
| *Thyasira (Parathyasira) neozelanica* | 1 | 4 | Doubtful Sound, NZ[E] | 376 |
| *Thyasira (Thyasira) dearboni* | 1 | 4 | Shetland Islands[C] | ? |
| *Thyasira (Thyasira)* n. sp. | 1 | 5 | W. Greenland[B] | 641 |
| *Thyasira (Thyasira) trisinuata* | 3 | 8–11 | off Daytona Beach, FL[C] | 185 |
| *Thyasira (Thyasira) sarsi** | 1 | 6 | White Sea[A] | 17–19 |
| | 2 | 7–14 | North Sea[B] | 200 |
| *Thyasira (Thyasira) flexuosa** | 3 | 5 | North Sea[B] | 200 |
| | 2 | 5 | Tatar Strait[A] | 30 |
| *Thyasira (Thyasira) gouldi* | 1 | 5 | W. Greenland[H] | 37 |
| | 1 | 5 | Faroe Islands[H] | 15 |
| | 1 | 5 | N. Greenland[H] | 10.5 |
| | 1 | 4 | Pechorskoye Sea[A] | 106 |
| | 1 | 4 | Kuril Islands[A] | 75 |
| *Thyasira (Thyasira) peregrina* | 1 | 4 | Pegasus Bay, NZ[E] | 505 |
| *Thyasira (Thyasira) bongraini* | 1 | 7 | Anvers Island, Antarctica[E] | 39 |
| *Thyasira (Thyasira) falklandica* | 1 | 18 | Anvers Island, Antarctica[E] | 5 |
| *Thyasira (Thyasira)* sp. | 2 | 3.5 | San Diego Trough[F] | 1215–1244 |
| *Conchocele excavata* | 1 | 15 | San Diego Trough[F] | 1250 |

[1] Asterisks (*) identify museum species that were also collected live.

[2] $n$ represents the number of specimens examined.

[3] A, Zoological Institute, Russian Academy of Sciences; B, Swedish Museum of Natural History; C, Smithsonian National Museum of Natural History; D, Royal British Columbia Museum; E, Museum of New Zealand; F, Scripps Institution of Oceanography Benthic Invertebrates Collection; G, Museum of Comparative Zoology, Harvard University; H, Zoological Museum, Copenhagen.

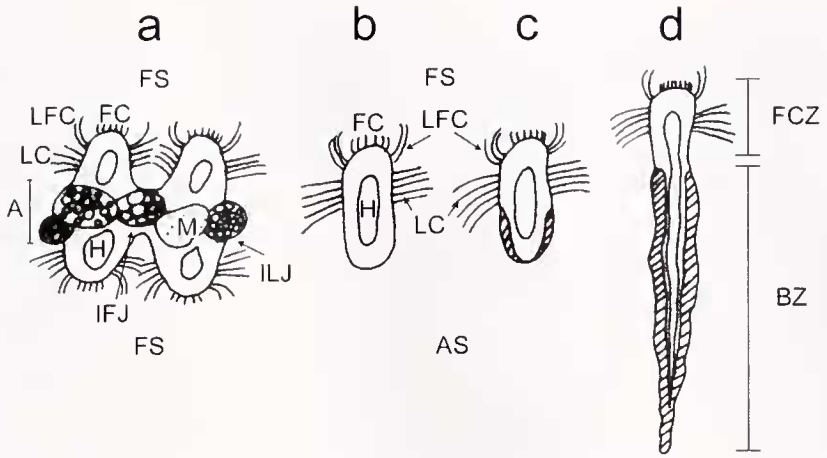


**Figure 1.** Schematic representation of transverse sections of gill filaments in the Thyasiridae. **(a)** Gill type 1. The ascending and descending arms of two neighboring filaments, interconnected by inter-filamentar (IFJ) and inter-lamellar (ILJ) tissue junctions. The frontal surface (FS) bears frontal cilia (FC), laterofrontal cirri (LFC), and lateral cilia (LC). In the abfrontal zone (A), several mucocytes (M) are present. At their dorsal end, the filaments are separate, as in (b). H, hemocoel. **(b, c)** Gill type 2. Filaments sectioned at the dorsal end of a demibranch. The frontal surface (FS) contains frontal cilia (FC), laterofrontal cirri (LFC), and lateral cilia (LC). Hatched areas at the abfrontal surface (AS) are occupied by symbionts in (c). H, hemocoel. **(d)** Gill type 3. Section through one arm of a gill filament. All cells in the bacteriocyte zone (BZ), below the frontal ciliated zone (FCZ), contain symbionts (hatched areas).

*Gill type 2*

Thyasirids within this group have simplified gill filaments with no apparent expansion of the abfrontal tissue (Fig. 1b, c; 3a). The frontal surface bears frontal cilia, laterofrontal cirri, and lateral cilia (Fig. 3a, b). Scanning electron micrographs (SEM) revealed the presence of several particles on the gill frontal surfaces, between filaments, and on the ventral food tract (Fig. 3b). Species with gill type 2 had either one or two demibranchs (Table 2); only specimens of the genera *Axinulus, Mendicula,* and *Adontorhina* had no outer demibranch (the presence of the gill axis on the outer side of the descending lamella of the lone demibranch confirms that it is the inner demibranch).

Transmission electron micrographs (TEM) revealed three types of cells in the abfrontal area of type 2 gill filaments: cells (C1) containing either several large mitochondria or, in *Mendicula ferruginea,* large electron-dense organelles (Fig. 3c, d); epithelial cells (C2) with more or less elongate microvilli (among which there may be symbionts — if so, these cells are called bacteriocytes); and mucocytes. When present, the mitochondria-rich cells (C1) seem to always be covered apically by thin extensions of C2 epithelial cells (Fig. 3c, d; 4a, b).

Only two species with gill type 2 were found to have symbiotic bacteria in the abfrontal zone (Table 2). These bacteria were located in the extracellular space between the apical cell membrane and microvillar extensions of the membrane (Fig. 4a, c). In TEMs, the density of symbionts within this extracellular space varied with species, and in *Thyasira (Parathyasira) equalis,* among individuals of a species (Fig. 4a, d). In one *T. (Parathyasira) equalis* specimen, symbionts were visible within bacteriocytes, perhaps having been taken up by endocytosis (Fig. 4b); in another specimen, careful survey of several filaments, on two grids prepared from separate gill areas, revealed a lack of symbionts (Fig. 4d). Other species with type 2 gills had no visible bacterial symbionts (Fig. 3c, d).

*Gill type 3*

Species with this gill type belong to the genera *Conchocele* and *Thyasira (Thyasira).* The gill filaments in these species are expanded abfrontally and have a distinct bacteriocyte zone (Fig. 1d; 5a–c). The frontal surface of the gills of *Conchocele excavata* and *T. (Thyasira)* sp. bears cilia typical of many suspension-feeding bivalves: frontal cilia, laterofrontal cirri, and lateral cilia can be seen (Fig. 5a, d). No cilia were visible on the surfaces of bacteriocytes or other abfrontal cells in these species (Fig. 5a, e).

In all specimens examined, the extracellular space occupied by bacterial symbionts was relatively large compared to the bacteriocyte cytoplasm (Fig. 5b, c). Symbionts were more abundant in association with the frontal-most bacteriocytes; their number (and occupied volume) decreased in cells having a more abfrontal position.

The cytological position of the symbionts was examined by transmission electron micrography. In all species where unambiguous observations could be made, the symbionts were extracellular—maintained in spaces delimited basally

**Table 2**

*Summary of data for the thyasirid species studied*

| Species | Gill type | Number of demibranchs | Symbiont abundance* | Maximum size† (mm) | Depth range† (m) |
|---|---|---|---|---|---|
| *Axinopsida orbiculata* | 1 | 2 | − | 8 (1) | 2–944 (2) |
| *Axinopsida serricata* | 1 | 2 | − | 8 (3) | 0–275 (3) |
| *Axinopsida subquadrata* | 1 | 2 | − | 3 (4) | 25–48 (4) |
| *Axinulus croulinensis* | 2 | 1 | + | 2.5 (1) | 24–3861 (5, 6) |
| *Axinulus eumyaria* | 2 | 1 | − | 2.5 (1) | 42–2663 (5) |
| *Axinulus* sp. | 2 | 1 | − | 1 (4) | 376 (4) |
| *Mendicula ferruginea* | 2 | 1 | − | 4.5 (1) | 40–4825 (3, 5) |
| *Mendicula pygmaea* | 2 | 1 | − | 2 (1) | 22–1470 (5, 7) |
| *Adontorhina cyclia* | 2 | 1 | − | 3 (3) | 12–3000 (3) |
| *Genaxinus debilis* | 2 | 2 | − | 3 (8) | 9–1000 (8,9) |
| *Genaxinus* sp. | 2 | 2 | − | 2 (4) | 298–403 (4) |
| *Thyasira (?) obsoleta* | 2 | 2 | − | 4 (1) | 24–2900 (5) |
| *Thyasira (Parathyasira) granulosa* | 2 | 2 | − | 10 (1) | 100–1800 (1) |
| *Thyasira (Parathyasira) equalis* | 2 | 2 | + | 8 (1) | 10–4734 (1,5) |
| *Thyasira (Parathyasira) neozelanica* | 2 | 2 | − | 4 (4) | 137–201 (10) |
| *Thyasira (Thyasira) dearboni* | 2 | 2 | − | 5 (8) | 222–1100 (8) |
| *Thyasira (Thyasira)* n. sp. | 2 | 2 | − | 5 (4) | 641 (4) |
| *Thyasira (Thyasira) trisinuata* | 3 | 2 | ++ | 18 (5) | 18–2359 (5, 11) |
| *Thyasira (Thyasira) sarsi* | 3 | 2 | ++ | 25 (1) | 50–340 (4, 12) |
| *Thyasira (Thyasira) flexuosa* | 3 | 2 | ++ | 12 (3) | 6–3000 (3, 13) |
| *Thyasira (Thyasira) gouldi* | 3 | 2 | ++ | 10 (1) | 5–732 (7) |
| *Thyasira (Thyasira) peregrina* | 3 | 2 | ++ | 5 (14) | 4–505 (4, 10) |
| *Thyasira (Thyasira) bongraini* | 3 | 2 | ++ | 7 (4) | 9–512 (15) |
| *Thyasira (Thyasira) falklandica* | 3 | 2 | ++ | 18 (15) | 5–344 (15) |
| *Thyasira (Thyasira)* sp. | 3 | 2 | ++ | 3.5 (4) | 1215–1244 (4) |
| *Conchocele excavata* | 3 | 2 | ++ | 24 (3) | 800–2520 (3) |

* ++, abundant symbionts; +, few symbionts; −, no symbionts seen.

† Numbers in parentheses indicate source from which the data were taken: (1) Oliver and Killeen, 2002; (2) Ockelmann, 1958; (3) Coan *et al.*, 2000; (4) present study; (5) Payne and Allen, 1991; (6) Verrill and Bush, 1898; (7) Aitken and Gilbert, 1996; (8) Cattaneo-Vietti *et al.*, 2000; (9) Dell, 1990; (10) Fleming, 1950; (11) Dall, 1899; (12) Zimmermann *et al.*, 1997; (13) López-Jamar and Parra, 1997; (14) Iredale, 1930; (15) Nicol, 1966.

by the bacteriocyte membrane and apically by microvilli (Fig. 6a–c). Extensions of the bacteriocyte cytoplasm among the symbionts were visible (Fig. 6b). In some individuals, material resembling lysed bacterial remains could be seen in the bacteriocytes (Fig. 6d, e). One *Thyasira (Thyasira) flexuosa* individual from off Long Beach contained viral particles, both within bacterial symbionts and in intracellular lysed remains (Fig. 6d).

Symbionts with different morphologies were observed: some appeared ovoid (Fig. 6a, b, e), whereas others were spherical (Fig. 6f) or rod-shaped (Fig. 6c). Empty spaces within the bacteria (Fig. 6b) may have been sulfur stores, which were washed out during specimen preparation (Vetter, 1985). In one specimen of *Thyasira (Thyasira) sarsi*, bacteria with different appearances were seen in association with different bacteriocytes (Fig. 6f). In one specimen of *T. (Thyasira) flexuosa* from a North Sea oil field, one or two electron-dense spheres were seen at the extremities of the symbionts (Fig. 6c); they appeared to be located between the external and internal bacterial membranes. Observation without prior osmium tetroxide fixation or heavy-metal staining revealed that these structures probably do not contain metals and may be organic (they were not electron-dense under those conditions).

## DISCUSSION

The results of this study improve our knowledge of thyasirid gills by covering a wider taxonomic range and nearly four times as many species as previously described. Overall, the data show an unusual amount of variation in gill anatomy and symbiont presence for bivalves at the family level, suggesting adaptations to different environments and lifestyles. Within genera and subgenera, gill characters are more conservative. The results appear to illustrate an evolutionary sequence, from a typical homorhabdic filibranch gill structure, with a few bacteria among abfrontal cell microvilli, to a modified gill with abfrontal thickening and a greater surface available to symbionts, the latter eventually becoming intracellular.

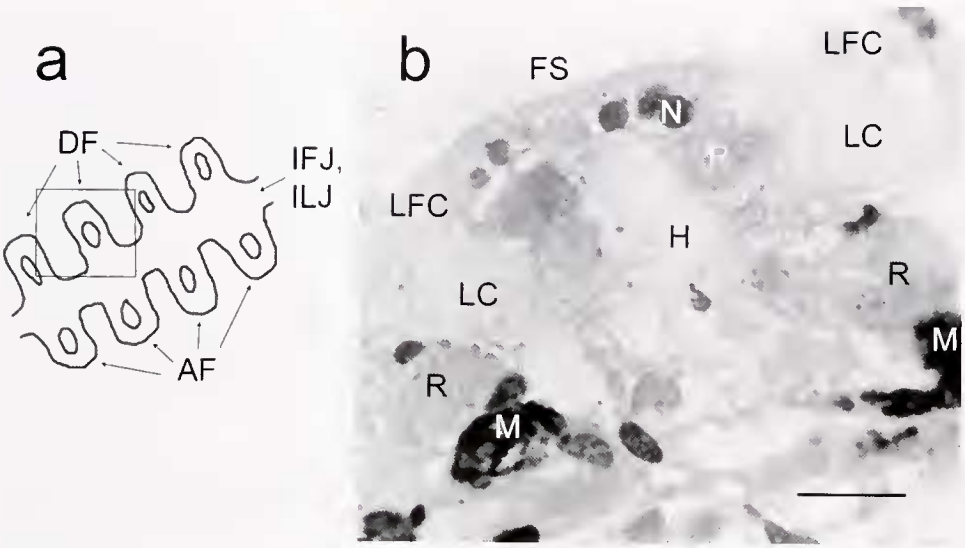


**Figure 2.** Type 1 gill filaments of *Axinopsida serricata*. **(a)** Diagram of a transverse section through the gill, with a square framing the location and orientation of the section depicted in (b). AF, ascending filaments; DF, descending filaments; IFJ, inter-filamentar junctions; ILJ, interlamellar junctions. **(b)** Light micrograph of a semi-thin section, showing nuclei (N) of cells at the frontal surface (FS), laterofrontal cirri (LFC) and lateral cilia (LC), and gill hemocoel (H). *Rickettsia*-like nodules (R) are visible among lateral ciliated cells and in the abfrontal zone, where several mucocytes (M) are also visible. Frontal cilia are not visible here, but were seen on other sections. Bar = 10 $\mu$m.

*Gill structure and symbiont presence*

The gill types are constrained within genera or subgenera: only *Axinopsida* species have gill type 1, and only *Conchocele*, *Thyasira (Thyasira)*, and *Maorithyas* (Fujiwara *et al.*, 2001) have gill type 3. Among the genera studied, *Thyasira* is most diverse, having type 2 and type 3 gills; for the most part, type 2 gills are found in the subgenus *Parathyasira*, and type 3 gills in the subgenus *Thyasira*. Whether gill structure may be used as a taxonomic character to distinguish subgenera of *Thyasira* is still unclear, since a global-scale taxonomy of this group based on other characters is not yet resolved.

Symbionts were identified in *Conchocele* and *Thyasira (Thyasira)* species (type 3), and in *Thyasira (Parathyasira) equalis* and *Axinulus croulinensis* (type 2). Judging from published figures, the symbiotic thyasirid *Maorithyas hadalis* has type 3 gills (Fujiwara *et al.*, 2001), and initial observations of *Conchocele bisecta* (data not shown) reveal even more complex gills, with cylindrical structures underlying the frontal ciliated layer, as in many lucinids (Distel and Felbeck, 1987). The gill types suggest an evolutionary sequence associated with the acquisition of symbionts, as described below.

Demibranch number is another variable character in thyasirids. From the present data, only species from the genera *Axinulus*, *Mendicula*, and *Adontorhina* do not have two demibranchs. This is not surprising given that demibranch number has been used as a taxonomic character (Payne and Allen, 1991). Although most symbiotic species from this survey have two demibranchs, *Axinulus croulinensis*, as well as a symbiotic thyasirid from the deep-sea (Southward, 1986), have only one. Thus, demibranch number seems unrelated to symbiont presence. As noted by Payne and Allen (1991), demibranch number is probably related to body size: thyasirids with only one demibranch are all less than 5 mm long. In bivalves, small body size and simple gill structure are common in species inhabiting the deep-sea (Allen, 1979). Hence, Payne and Allen (1991) suggested that, in thyasirids, the loss of a demibranch and small body size are adaptations for deep-sea life. A survey of thyasirid depth distribution (Table 2) reveals that many of the larger species can nonetheless be found at bathyal depths; but among asymbiotic thyasirids, most of the deep-sea dwelling species are small and have only one demibranch.

Of the thyasirid species studied here, those with symbionts are larger and live at somewhat shallower depths than those without symbionts (Table 2). The larger size could be due to their having a greater nutritional input than strict suspension-feeders, especially in the deep-sea, where particulate food is less abundant. If the symbionts described here are chemoautotrophic sulfide-oxidizing bacteria, as in other thyasirids (Dando and Southward, 1986), the apparent preference of symbiotic thyasirids for shallower depths may be based on a bacterial requirement for reduced sulfur, which is more common in sediments on the continental

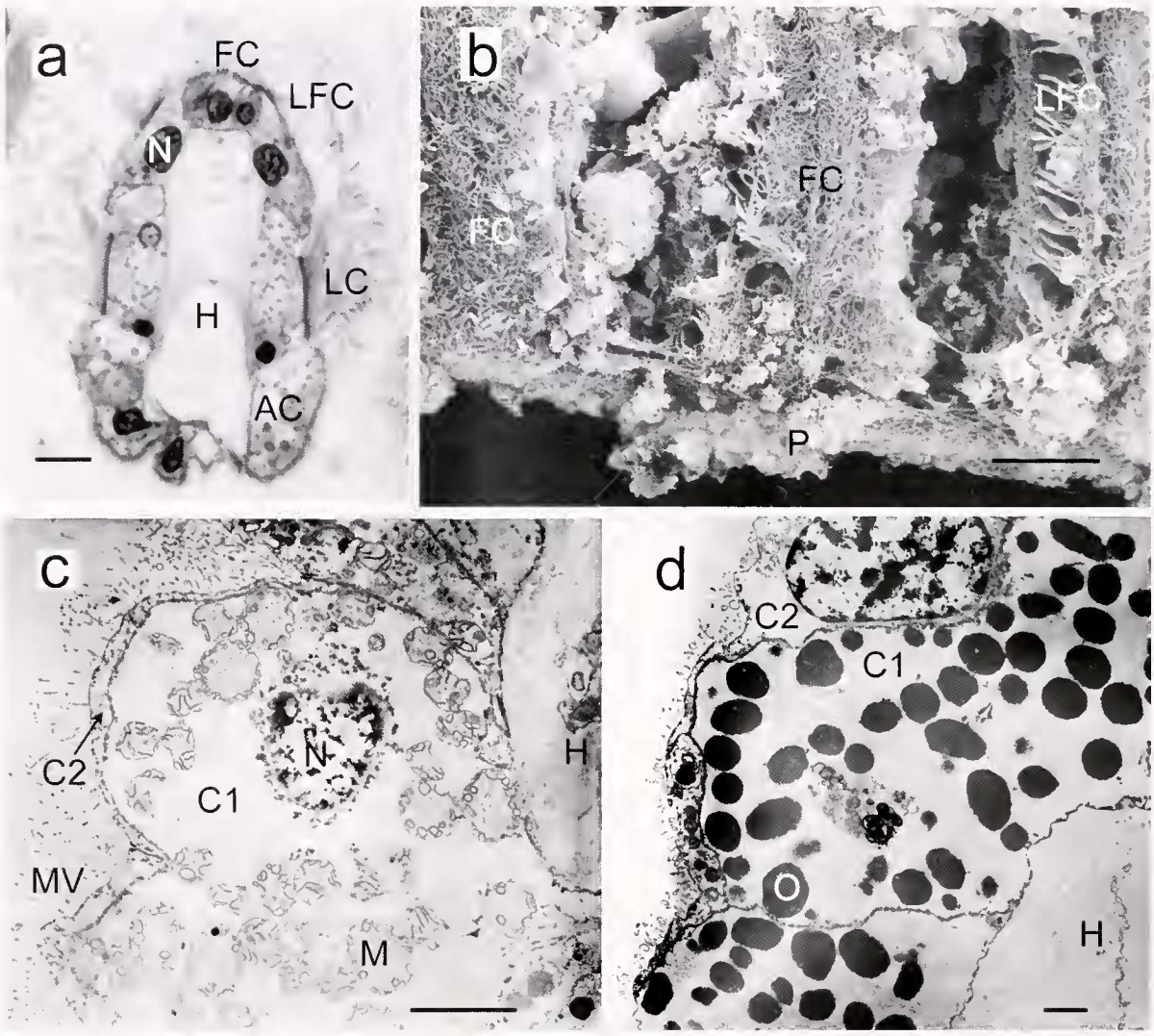


**Figure 3.** Light and electron micrographs of Type 2 gill filaments. **(a)** *Thyasira (Parathyasira) equalis.* Light micrograph of a semi-thin, transverse section of a gill filament, showing frontal cilia (FC), laterofrontal cirri (LFC), lateral cilia (LC), hemocoel (H), and abfrontal cells (AC). N, nucleus. **(b)** *Adontorhina cyclia.* SEM of the ventral extremity of three gill filaments. Frontal cilia (FC) and laterofrontal cirri (LFC) are visible. Numerous particles can be seen on the frontal tracts, between filaments, and along the ventral food tract (P). **(c)** *T. (?) obsoleta.* TEM of cells in the abfrontal zone of a gill filament. Thin cells (C2) with microvilli (MV) cover the apical surface of cells (C1) with numerous mitochondria (M). N, nucleus; H, hemocoel. **(d)** *Mendicula ferruginea.* TEM of cells (C1) with electron-dense organelles (O); these cells are covered at their apical end by thin cells (C2). H, hemocoel. Bars: (a) = 25 μm; (b) = 20 μm; (c, d) = 1 μm.

shelf, where organic matter is more abundant. At deeper sites, thyasirids (some of which may have symbionts) have been found at hydrothermal vents (Gebruk *et al.*, 2000), at cold seeps (Clarke, 1989; Lewis and Marshall, 1996; Imhoff *et al.*, 2003), and in oxygen-minimum zones (L. Levin, Scripps Institution of Oceanography; pers. comm.), where sulfide is more abundant. Elsewhere in the deep-sea, symbiotic thyasirids could rely on sparser amounts of sulfide: even at coastal depths, symbiotic thyasirids are often found in sediments where sulfide levels are low or undetectable (Dando and Southward, 1986). The ability of symbiotic thyasirids to mine for sulfide by using their superextensible foot allows them to access patches of sulfide in such environments (Dufour and Felbeck, 2003).

Symbiont density varied among specimens of *Thyasira (Parathyasira) equalis* (Fig. 4a, b, d). This may be related to seasonal or spatial variations in available sulfide in the sediment, differentially affecting the fitness and growth of symbionts among thyasirid individuals. Dando and Spiro (1993) noted that *T. (Thyasira) sarsi* and *T. (Parathyasira) equalis* exhibited seasonal variability in $^{13}C/^{12}C$ ratios, and concluded that their nutritional dependence upon their sym-

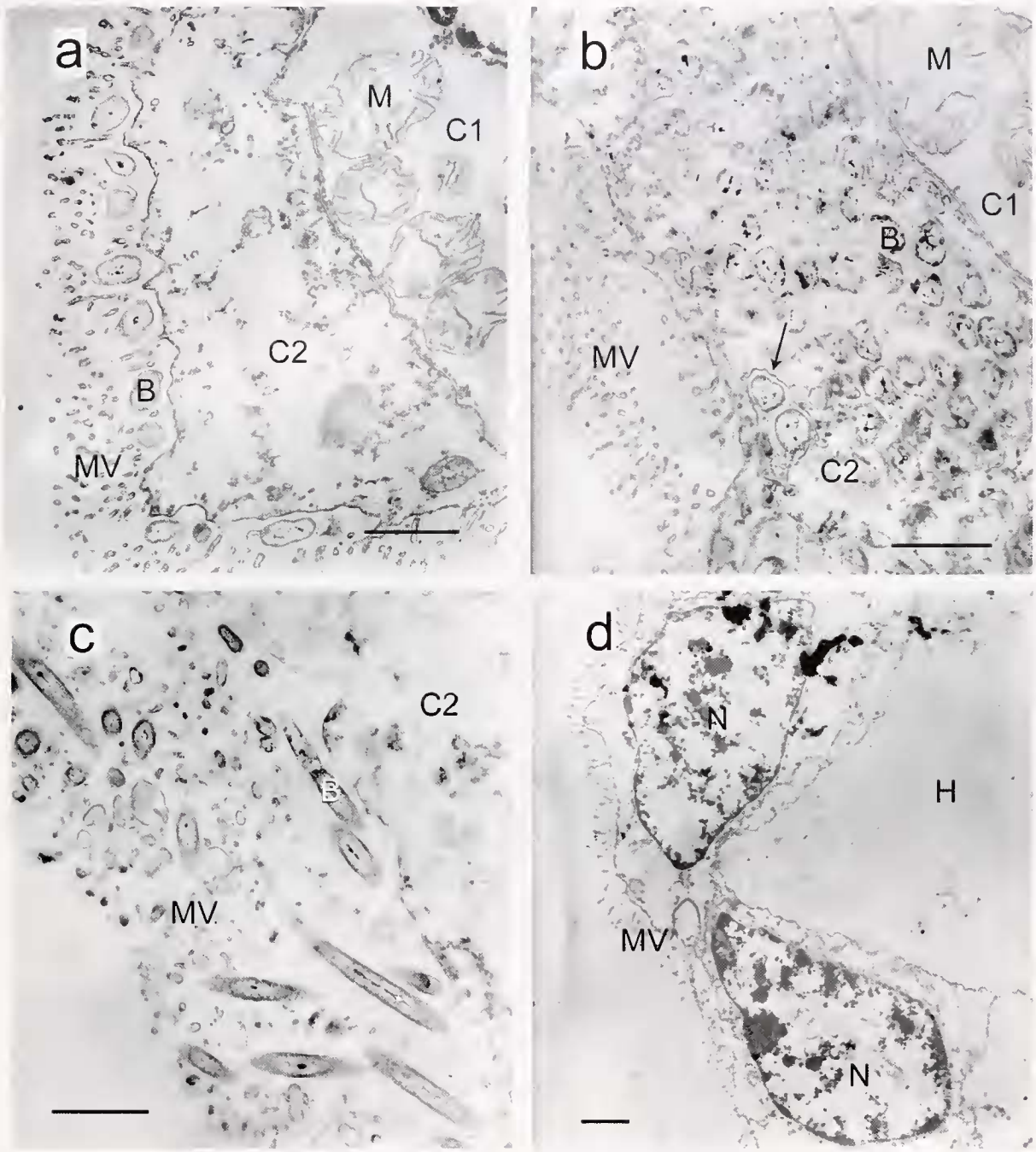


**Figure 4.** TEM of cells in the abfrontal zone of thyasirids with gill type 2. **(a)** *Thyasira (Parathyasira) equalis.* Bacteria (B) are present among the microvilli (MV) of thin epithelial cells (C2) in the abfrontal zone. Underlying cells (C1) contain several mitochondria (M). **(b)** *T. (Parathyasira) equalis.* Thin epithelial cell (C2), with degrading bacteria (B) in the cytoplasm (none are visible among the microvilli [MV]). Arrow points to symbionts within a vacuole. Mitochondria (M) are visible in the underlying cell (C1). **(c)** *Axinulus croulinensis.* Apical end of an abfrontal cell (C2), with bacterial symbionts (B) among the microvilli (MV). **(d)** *T. (Parathyasira) equalis.* Abfrontal cells in an individual without symbionts. H, hemocoel; MV, microvilli; N, nucleus. Bars = 1 μm.

bionts varied with changing sulfide content in the sediment. Perhaps this change in carbon isotope ratios is caused, not by a lower rate of symbiont digestion when organic matter is low, but rather by the sparseness of symbionts at these times. Although not reported, symbiont density may vary similarly in other chemosymbiotic bivalves; moreover, spe-

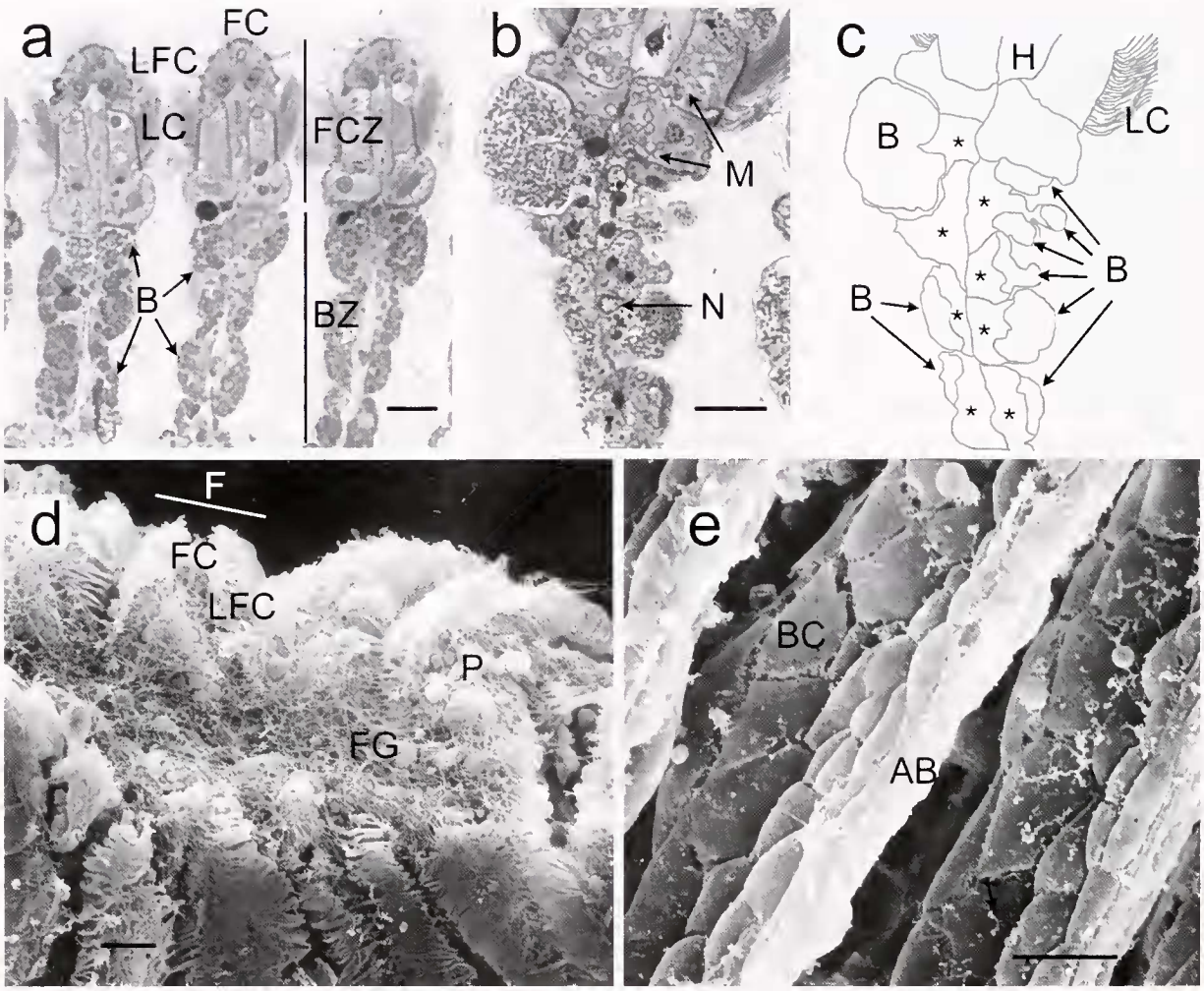


**Figure 5.** Light and electron micrographs of type 3 gill filaments. **(a)** Light micrograph of a semi-thin, transverse section of three gill filaments of *Thyasira (Thyasira) flexuosa*, showing frontal ciliated zone (FCZ) and bacteriocyte zone (BZ). Frontal cilia (FC), laterofrontal cirri (LFC), and lateral cilia (LC) are visible. Darker stained areas within bacteriocytes are symbionts (B). **(b,c)** Light micrograph of a semi-thin, transverse section and diagram of a gill filament of *T. (Thyasira) flexuosa*. Asterisks show the cytoplasm of bacteriocytes, at the apical end of which are kept the bacteria (B). Mitochondria (M) are visible in cells of the frontal ciliated zone. H, hemocoel; LC, lateral cilia; N, nucleus. **(d)** SEM of the ventral food groove (FG) of gill filaments (F) of *T. (Thyasira)* sp., revealing frontal cilia (FC) and rows of laterofrontal cirri (LFC). P, particles. **(e)** SEM of the abfrontal end (AB) in the bacteriocyte zone of *T. (Thyasira)* sp. gill filaments. The outlines of individual bacteriocytes (BC) are visible. Bars: (a) = 35 μm; (b) = 25 μm; (d, e) = 20 μm.

cies observed here as having no symbionts could in fact possess them at different times or locations. The implication of this variation in symbiont density is that the symbiosis may be facultative in some thyasirids, serving more as a nutritional supplement than a primary food source.

The symbionts themselves showed variation in size and structure: seen in sections, some were rod-shaped, while others appeared ovoid or spherical. The various morphologies may indicate that the symbionts in different thyasirids are different species; further examination using molecular techniques would resolve this issue.

One specimen of *Thyasira (Thyasira) sarsi* had what appeared to be two morphotypes of symbionts within two neighboring bacteriocytes; this would not be the first report of two different symbionts within a thyasirid, as they were also seen in *Maorithyas hadalis* (Fujiwara *et al.*, 2001) and in two unidentified deep-sea thyasirids (Southward, 1986).

### *The evolution of chemosymbiosis*

The different gill structures observed in the family Thyasiridae, when related to the presence and cytological location of symbionts, are suggestive of the pathways of gill evolution in chemosymbiotic autobranch bivalves. It is

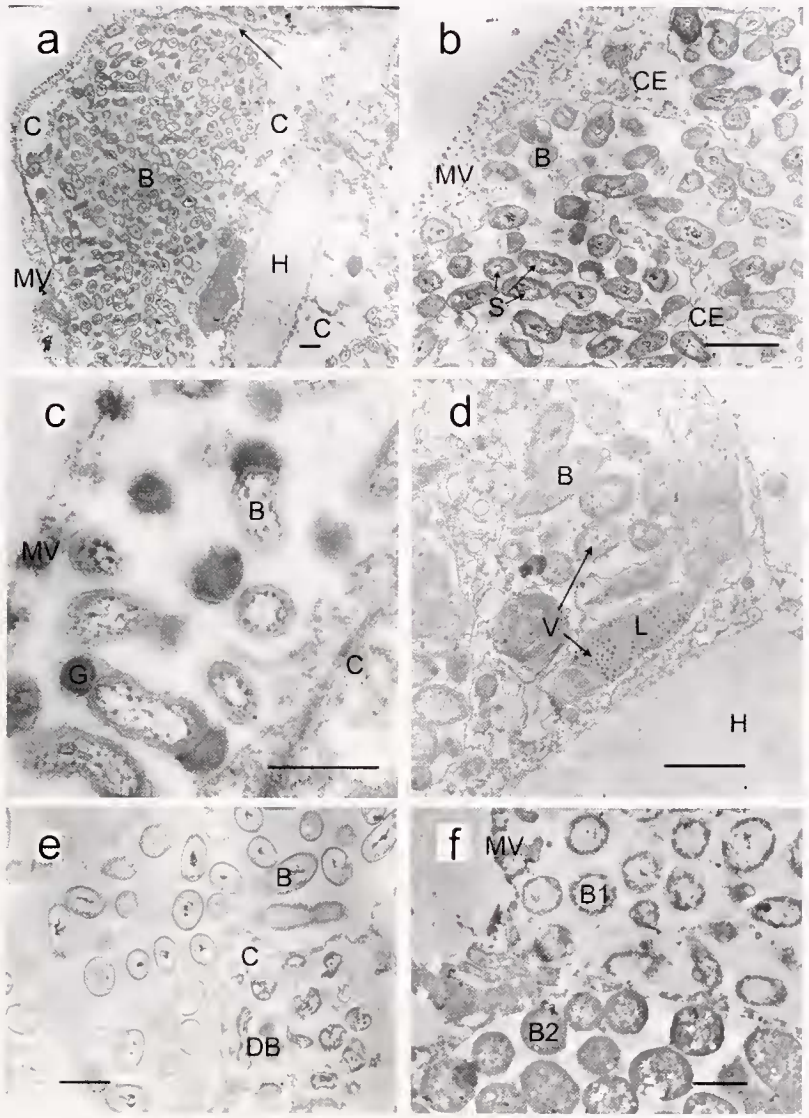


**Figure 6.** TEM of bacteriocytes in thyasirids with gill type 3. **(a)** Oblique section of *Thyasira (Thyasira) flexuosa* bacteriocytes. Bacteria (B) are extracellular and distinct from cytoplasm (C). Arrowhead points to a cytoplasm extension. H, hemocoel; MV, microvilli. **(b)** Apical end of a *T. (Thyasira) flexuosa* bacteriocyte, showing detail of cytoplasm extension (CE), microvilli (MV), and bacteria (B). Translucent areas within bacteria may have contained sulfur deposits (S), which washed out during TEM preparation. **(c)** Bacterial symbionts (B) in *T. (Thyasira) flexuosa* from an oil field in the North Sea. Electron-dense granules (G) are visible at one or both ends of the rod-shaped bacteria. C, bacteriocyte cytoplasm; MV, microvilli. **(d)** The basal end of a *T. (Thyasira) flexuosa* bacteriocyte, with lysosomes (L) containing the remains of bacteria (B) and viruses (V). H, hemocoel. **(e)** Bacteriocyte cytoplasm (C) of *T. (Thyasira) sarsi* and associated bacteria (B). DB, degrading bacteria. **(f)** Two neighboring bacteriocytes in *T. (Thyasira) sarsi*, each cell having a different morphotype of bacteria (B1 and B2). MV, microvilli. Bars: (a, b, d–f) = 1 μm; (c) = 0.5 μm.

plausible that, with time, symbionts progressed from an extracellular location to an intracellular one (Fig. 7), and that gills became more elongate.

In all the thyasirids observed, the symbionts were normally located extracellularly, within spaces delimited by the cell membrane and microvilli (except when presumably taken up by endocytosis). This is believed to be a simple state (Smith, 1979; Hickman, 1994; Cavanaugh, 1994), with bacteria being taken up by endocytosis and digested within the host epithelial cells (Le Pennec *et al.*, 1988). Evidence for this digestion include the presence of lysosomal bodies with accumulated bacterial membranes within the cells, which have been seen, not only in thyasirids, but also in mytilids, vesicomyids, lucinids, and solemyids with endosymbionts (Fiala-Médioni *et al.*, 1986; Fiala-Médioni and Le Pennec, 1987; Le Pennec *et al.*, 1988; Fisher, 1990;

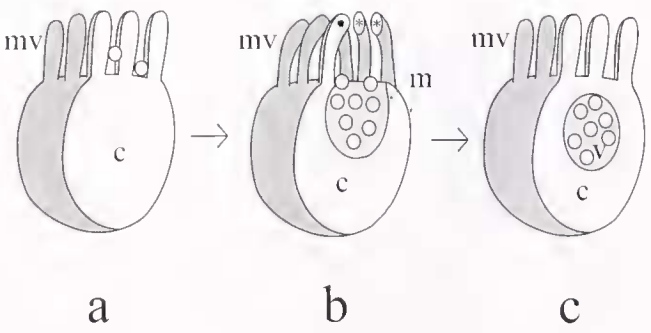


**Figure 7.** Diagram, based on thyasirid gill data, showing the evolution of symbiosis from an extracellular to an intracellular mode. Each diagram represents a bacteriocyte, sectioned along the apical-basal plane (revealing, in white, what is typically seen in transverse sections of gills). Bacteria are represented by small circles. **(a)** Bacteriocyte in thyasirids with gill type 2, with bacteria present among the microvilli (mv), but separate from the cytoplasm (c). **(b)** Bacteriocyte in a gill type 3, with bacteria (circles) still maintained outside the cytoplasm (c), but in a space delimited by the bacteriocyte membrane (m), and microvilli (mv). In sections, the microvilli appear either entire (star) or partial (asterisks). **(c)** Bacteriocyte in the type 3 gill of *Maorithyas hadalis* (or other bivalves with endosymbionts) maintained in a vacuole (v), within the cytoplasm (c). mv, microvilli.

Barry *et al.*, 2002). The endocytosis of bacteria is not a phenomenon restricted to epithelial cells of the gills of chemosymbiotic bivalves; it is an inherent defense mechanism of almost all eukaryotic cells (Silverstein *et al.*, 1977). The likelihood of bacteria passing between gill filaments and reaching the abfrontal surfaces without prior interception by laterofrontal cilia appears high, given the relatively large space between filaments, as seen by naked eye upon dissection, and on scanning electron micrographs (Fig. 3b; although the position of particles on these specimens may be artifactual, the wide spaces between filaments, coupled with laterofrontal cirri that are not abnormally large, suggest that bacteria can easily reach abfrontal surfaces).

In the species *Maorithyas hadalis*, as well as in some species of *Bathymodiolus*, and in the symbiotic gastropods *Ifremeria nautilei* and *Alviniconcha hessleri*, apically situated vacuoles containing bacteria often have an open connection to the external water (Le Pennec and Hily, 1984; Endow and Ohta, 1989; Windoffer and Giere, 1997; Dubilier *et al.*, 1998; Fujiwara *et al.*, 2001). This arrangement was suggested to be an intermediate state between extracellular and intracellular symbionts (Windoffer and Giere, 1997), where only bacteria in vacuoles close to the apical end of bacteriocytes are in contact with seawater (Endow and Ohta, 1989). In the gills of symbiotic gastropods, the bacteria are enclosed within a vacuolar network, which may allow exchange with external water (Windoffer and Giere, 1997). Extracellular bacteria may receive some benefit (such as dissolved gases or nutrients) from being in contact with flowing seawater. On the other hand, when symbionts are enclosed by host-cell microvilli, the latter may exercise some control on the nature of the fluid bathing the symbionts.

The microvilli of symbiotic thyasirids appear more elongated than those of typical bivalve gill epithelial cells (Morse and Zardus, 1991). Elaborate microvillar layers are typical of surfaces involved in symbiosis, such as the light organ of squids (Montgomery and McFall-Ngai, 1993; McFall-Ngai, 1998) or enteric epithelia (Woolverton *et al.*, 1992). Interestingly, some of the nonsymbiotic thyasirids had similarly elongated microvilli; this suggests that they may have the ability to retain bacteria, and that an examination of additional specimens might reveal symbionts in some specimens.

In thyasirids, the uptake of symbionts within epithelial cells is predated by the abfrontal development of gill filaments, which increases the space available for bacterial colonization. Only in the thyasirid *Maorithyas hadalis* have intracellular symbionts been observed (Fujiwara *et al.*, 2001); this species has a type 3 gill. Perhaps intracellular symbionts are present in other species in the genus *Maorithyas*, as well as in certain *Conchocele;* the structure of the gill of *C. bisecta*, with its bacteriocyte cylinders, certainly suggests a more advanced state. The presence of symbionts in the simpler type 2 gill of *Thyasira (Parathyasira) equalis* suggests that this species has acquired its symbionts relatively recently.

The abfrontal elaboration of gill filaments appears to be a more efficient way to increase the overall space available for bacterial housing, compared to an increase in the number of unmodified filaments per gill: in the second case, the bivalves have to elaborate and maintain not only extra bacteriocytes, but also additional frontal ciliated zones.

## ACKNOWLEDGMENTS

I am grateful to H. Felbeck, J.B.C. Jackson, N.D. Holland, and W. Newman at the Scripps Institution of Oceanography (SIO), and K. Roy at the University of California, San Diego, for their advice and support. P.V. Scott at the Santa Barbara Museum of Natural History provided assistance with thyasirid identifications. D. Cadien at the County Sanitation Districts of Los Angeles County; S. Parra and I. Frutos at the Instituto Español de Oceanografía, in La Coruña; T. Hoisæter, P. Johannessen, and staff at the University of Bergen and the Marine Biological Station, Espegrend, were involved in the collection of specimens. F. Gaill, J.-P. Lechaire, and G. Frebourg at the Université Pierre et Marie Curie, Paris; L. Rudee at the Materials Sciences Department at the University of California, San Diego; and S. Barlow at the San Diego State University provided transmission electron microscopy facilities and resources. C. Graham from the SIO Analytical Facility assisted with scanning electron microscopy. The American

Malacological Society and the SIO provided financial support.

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